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Derivatives of 8-Hydroxy-2-methyl-quinoline are Powerful Prototypes for Zinc Sensors in Biological Systems

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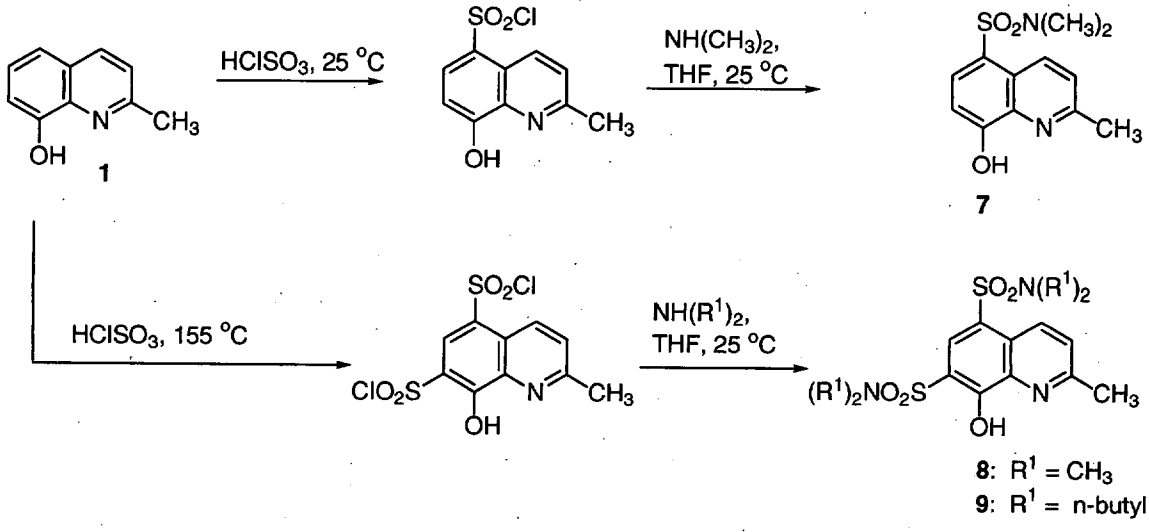
Synthesis of Oxn Derivatives

Figure S1: Methodology used to prepare sulfonamide derivatives of 8-Hydroxy-2-Methyl-quinoline.



8-Hydroxy-2-methyl-quinoline-5-sulfonyl chloride.

8-hydroxy-2-methyl-quinoline (2.0 g, 12.6 mmol) was dissolved in chlorosulfonic acid (10 ml) and stirred at 25 °C for 2 hours, after which time the reaction mixture was added to a 2 L separatory funnel containing a slurry of ice in brine (500 ml) and a layer of dichloromethane (DCM, 500 ml). The layers were shaken briefly (10 – 20 seconds) and the DCM layer run into a flask containing a bed (5 - 10 g) of potassium carbonate to remove residual water. The resulting solution was filtered and solvent removed by rotary evaporation to leave the crude product as a yellow powder (2.8 g, 87%), which was used without further purification in the synthesis of **4** and **7**. TLC $R_f = 0.63$ (silica, $CHCl_3$/MeOH, 4:1) $R_f = 0.39$ (silica, ethyl acetate). ^{1}H NMR ($CDCl_3$, 300 MHz) 9.04 (d, J = 8.7, 1H), 8.35 (d, J = 8.4, 1H), 7.68 (d, J = 8.7, 1H), 7.29 (d, J = 8.4, 1H), 2.89 (s, 3H). HRMS (+ESMS): Calcd for $C_{10}H_9NO_3ClS$ $[M+H]^+$ 257.9993, Found 258.0061. *Anal. Calcd.* for $C_{10}H_8NO_3Cl$: C,46.61; H, 3.13; N, 5.44; Cl, 13.76. Found: C, 46.82; H, 3.02; N, 5.25; Cl, 13.39.

8-Hydroxy-2-methyl-quinoline-5-sulfonic acid (4).

This species has been prepared previously by treatment of 8-hydroxy-2-methyl-quinoline with sulfuric acid.[1] For the study reported here, a sample of 8-hydroxy-2-methyl-quinoline-5-sulfonyl chloride (0.20 g, 0.78 mmol) was stirred in water (10 ml) for 48 hours. The solution was reduced to about 2 ml and the pale yellow solid product collected by vacuum filtration, washed with cold water and dried under vacuum (0.16 g, 86%). The product obtained was consistent with the product characterized in the literature.[1]

8-hydroxy-2-methyl-quinoline-5-(N,N-dimethyl)sulfonamide (7).

N,N-dimethylamine was bubbled through dry tetrahydrofuran (THF, 25 ml) for 15 minutes before 8-hydroxy-2-methyl-quinoline-5-sulfonyl chloride (0.2 g, 8.27 mmol) was added in small portions (*ca* 10 mg/addition) over 3 hours at 25 °C. The reaction mixture was stirred a further hour, during which time dimethylamine was continually bubbled through the reaction mixture. The solvent was removed by rotary evaporation and some

excess dimethylamine was removed by dissolving the sticky residue in dichloromethane (50 ml) and rotary evaporation (repeated three times). The crude product was obtained in almost quantitative yield (> 95%), but, for analytical experiments, was purified by flash chromatography on silica (silica gel, dimensions 2.5 x 25 cm, ethylacetate/hexane, 10:90, 10 ml fractions). Product is an off white powder (0.084 g, 45%). mp 133-134 °C R_f = 0.65 (silica, CH_3Cl/MeOH, 4:1). R_f = 0.49 (silica, ethyl acetate). ^1H NMR ($CDCl_3$, 300 MHz): 8.97 (d, J = 8.9, 1H), 8.09 (d, J = 8.3, 1H), 7.47 (d, J = 8.9, 1H), 7.17 (d, J = 8.3, 1H), 2.76 (s, 3H), 2.75 (s, 6H). ^{13}C NMR ($CDCl_3$, 75.46 MHz): 158.0, 156.4, 137.7, 134.7, 132.4, 124.7, 123.8, 121.8, 108.3, 37.8, 25.1. FTIR (film, cm^{-1}) 3336, 2962, 2922, 2849, 2272, 1598, 1565, 1503, 1471, 1399, 1327, 1270, 1248, 1705, 1185, 1154, 1119, 959, 836, 717, 658, 637, 613. HRMS (+ESMS): Calcd for $C_{12}H_{14}N_2O_3S$ $[M+H]^+$, 267.0805; Found 267.0831.

Note 1: Small quantities of white suspensions formed in all THF solutions of sulfonamide preparations reported here. The suspensions were attributed to the formation of salts of the amine and/or the phenolic oxygen with product HCl.

Note 2: By-products in these reactions gave mass spectral and ^1H NMR data that were consistent with dimers formed by addition of the sulfonyl group of one molecule to the pyridine nitrogen of another. Conducting the synthesis under conditions in which the amine was present in vast excess to the dilute, unreacted sulfonyl chloride significantly reduced by-product formation.

8-Hydroxy-2-methyl-quinoline-5,7-bis(N,N-dimethyl)sulfonamide (8).

A sample of 8-Hydroxy-2-methyl-quinoline-5,7-disulfonyl chloride[1] (0.2 g, 0.56 mmol) was added in small portions (*ca* 10 mg/addition) over 3 hours at 25 °C, to a solution of N,N-dimethylamine in dry THF (25 ml, 2 M) and the reaction mixture was left to stir overnight. The solvent was removed from the reaction mixture by rotary evaporation and some excess dimethylamine was removed by dissolving the sticky residue in dichloromethane (50 ml) and rotary evaporation (repeated three times). The crude off-white product was recovered in quantitative yield but, for analytical experiments, was

recrystallized by dissolving the residue in a minimum volume of 6 M HCL, dilution with water and storage at 4 °C (repeated three times) (0.066g, 32%). mp 188-189 °C. R_f = 0.51 (silica, $CHCl_3$/MeOH, 4:1). R_f = 0.14 (silica, ethyl acetate). ^{1}H NMR ($CDCl_3$, 300 MHz): 9.04 (d, J = 8.8, 1H), 8.47 (s, 1H), 7.61 (d, J = 8.8, 1H), 2.93 (s, 6H), 2.83 (s, 3H), 2.81 (s, 6H). ^{13}C NMR (d_6-DMSO, 75.46 MHz): 161.8, 157.5, 138.8, 136.2, 133.8, 117.7, 113.3, 38.4, 37.8, 23.8. FTIR (film, cm^{-1}) 3252, 2959, 2924, 1600, 1558, 1496, 1456, 1428, 1335, 1258, 1199, 1163, 1141, 962, 776, 718, 622. HRMS (+ESMS): Calcd for [M+H]+, $C_{14}H_{19}N_3O_5S_2$ 374.0846; Found 374.0858.

8-Hydroxy-2-methylquinoline-5,7-bis(N,N-dibutylamine)sulfonamide (9).

A sample of 8-hydroxy-2-methyl-quinoline-5,7-disulfonylchloride (0.2 g, 0.56 mmol) was added in small portions (*ca* 10 mg/addition) over 3 hours at 25 °C, to a solution of N,N-dibutylamine (50 ml) in dry THF (50 ml) and the reaction mixture was left to stir overnight. The solvent was removed from the reaction mixture by rotary evaporation and the reaction mixture added to a 1 L separatory funnel containing a slurry of ice in 6 M HCl (400 ml) and DCM (300 ml). After extraction, the DCM layer was washed with 1 M HCl (200 ml x 7), dried ($MgSO_4$) and the solvent removed to result in a yellow oil. This was dissolved in a minimum volume of methanol, water added to the first signs of permanent turbidity and cooled at 5 °C overnight. The product, cream colored needles, was collected and allowed to air dry (0.18 g, 0.33 mmol, 54%). A second crop was obtained by addition of water (ca 1 ml) to the filtrate. R_f = 0.85 (silica, $CHCl_3$/MeOH, 4:1). R_f = 0.73 (silica, ethyl acetate). ^{1}H NMR ($CDCl_3$, 300 MHz): 8.67 (d, J = 8.9, 1H), 8.29 (s, 1H), 7.43 (d, J = 8.9, 1H), 3.14 (m, 8H), 2.66 (s, 3H), 1.40 (m, 8H), 1.13 (m, 8H), 0.73 (m, 12H). ^{13}C NMR ($CDCl_3$, 75.46 MHz): 158.6, 152.9, 137.3, 134.2, 128.1, 125.4, 125.2, 124.0, 118.8, 47.6, 46.5, 30.49, 29.9, 24.3, 19.5, 19.4, 13.3, 13.2. HRMS (+ESMS): Calcd for [M+H]+, $C_{26}H_{43}N_3O_5S_2$ 542.2723; Found 542.2813.

Spectrophotometric Characterization of Quinoline Derivatives

All buffers and stock solutions of EDTA, NaOH, HCl and metal ions were prepared using analytical grade or high purity chemicals (Aldrich) and Milli-Q (Millipore) treated water. The buffer solutions were treated by passage through a column of Chelex resin (Na+ form, Bio Rad) to remove trace metal impurities. After Chelex treatment the pH of the buffer was adjusted to pH 7.0 if necessary. The concentrations of stock solutions of metal chloride salts were determined by titration with standardized solutions of EDTA (Aldrich) in the presence of an appropriate metallochrome indicator.[2] The pH of the stock metal ion solutions was adjusted to pH 3.0 (1 mM HCl) after standardization. Stock solutions of the quinoline derivatives were prepared using spectrometric grade dimethylsulfoxide (DMSO) or dioxane (Aldrich). Trace metal ion impurities in derivatives **4**, **5**, **7-9** generated fluorescence signal in the absence of added zinc. This could be significantly reduced by recrystallizing the derivatives from high purity HCl (double distilled into quartz, from Seastar) and Milli-Q treated water.

UV-visible and fluorescence assays with Oxn derivatives (0.1 – 50 μM) were performed in 50 mM HEPES, 150 mM NaCl (pH 7.01). Addition of Zn^{2+} to solutions of Oxn derivatives resulted in consistent changes in UV-visible and fluorescence spectra, Figure S2. The red shifts in absorbance bands of disulfonamide ligands (**8**, **9**) on addition of Zn^{2+} (5-10 nm) were less than that observed in spectra of other derivatives. The stoichiometry of the complexes formed on addition of Zn^{2+} to solutions of Oxn derivatives were analyzed using Job's method of continual variance.[3] The experiments were conducted such that the value of total [derivative] + [Zn^{2+}] was constant and in the range of 5 – 50 μM. The data demonstrated that the association of metal ion for the ligands was weak and that a mixture of 1:1 and 1:2 complexes existed at pH 7.0.

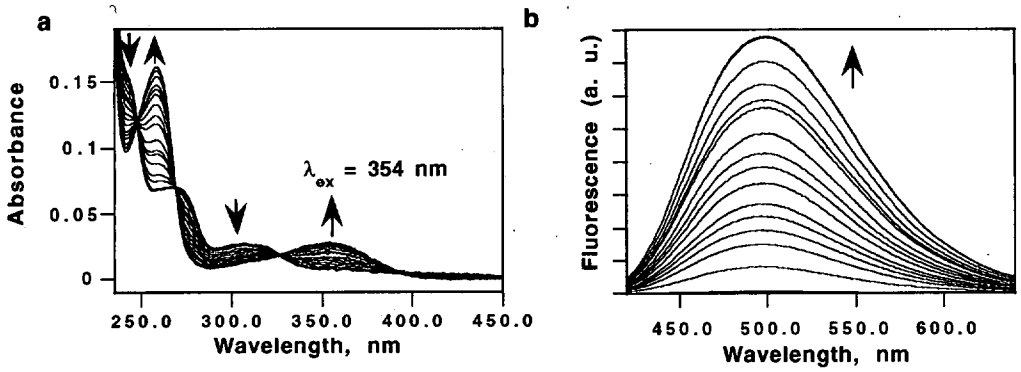


Figure S2. a) Titration of Zn^{2+} (0 – 700 μM) into a solution of **7** (5.1 μM) results in redshifted absorbance bands in the UV-visible spectra. b) Irradiation at the new absorbance band at 354 nm results in fluorescence emission at 499 nm.

For the purposes of comparison, the $[Zn(II)]_{free}$ at half fluorescence maximal intensity (half the ligand is complexed to the metal ion) in the pseudo-biological conditions were determined using a previously described method.[4] Titration of $ZnCl_2$ into buffered solutions of **7** was monitored by both UV-vis and fluorescence spectroscopy. Titration of $ZnCl_2$ into buffered solutions of **8** was monitored by fluorescence spectroscopy. Derivative **9** was not sufficiently soluble in the aqueous, buffered solutions to allow a value of K_D to be defined. However, initial experiments indicate that the affinity of this species for Zn(II) is similar to that of **8**.

The quantum yields of the quinoline derivatives in 50 mM HEPES, 150 mM NaCl (pH 7.01) were calculated using quinine sulfate in 0.1 M H_2SO_4 (Molecular Probes) as a standard, allowing for the wavelength-dependent changes of quantum yield for this molecule.[5] An uncertainty of 10% has been associated with this value, and the quantum yields of the quinoline derivatives are assumed to have an error of not less than 10% as well. Solutions of the quinoline derivative (1 – 5 μM) and $ZnCl_2$ (to ensure saturation of the ligand: 0.5 – 1 mM) in the HEPES buffer were used to collect the steady state emission data required for the quantum yield calculations. In calculating the quantum yields the refractive index of the HEPES buffer solution was assumed to be equivalent to the refractive index of 0.1 M H_2SO_4.

pH Dependence of Fluorescence.

A pH titration of cell permeable derivative **9** in the presence of 1 mM EDTA or 1 mM $ZnCl_2$ was obtained to demonstrate the influence of intracellular fluctuations in pH on the fluorescence response of the fluorophore. In the pH range 4-8 measured fluorescence emission was virtually constant in both titrations, Figure S3.

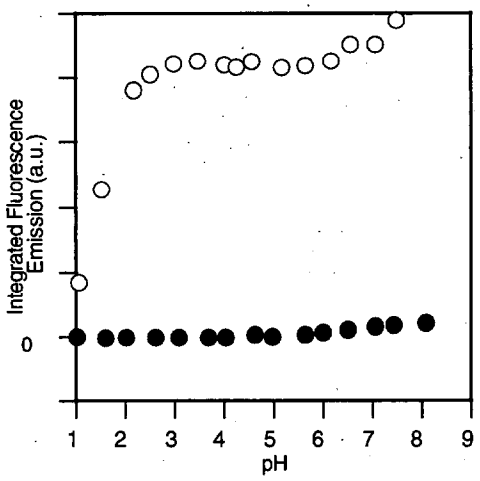


Figure S3. pH dependence of the fluorescence emission from **9** (1 μM, 50 mM HEPES, pH 7.04, containing 150 mM NaCl and 5% dmso) in the pH range 1 – 8. Solid circles: titration performed in the presence of 1 mM EDTA. Open circles: titration performed in the presence of 1 mM $ZnCl_2$. Titration beyond pH 8 was complicated by precipitation (most likely the metal hydroxide).

Metal Ion Selectivity of Fluorescence

The fluorescence response of the fluorophores to the addition of a range of metal ions, including Ca(II), Mg(II), Al(III), Fe(III), Co(II), Ni(II), Cu(II) and Cd(II) has been investigated (the ligands are not responsive to addition of Na^+ and K^+; solution phase experiments are performed in 150 mM NaCl). All species respond in a similar manner; the response of **9** is summarized in Figure S4. Figure S4a describes the fluorescence of **9** to an addition of 10 equivalents of a variety of metal ions, while Figure S4b describes the fluorescence of **9** to a mixture of 10 equivalents of Zn(II) and 10 equivalents of another

metal ion. The response is consistent with both the Irving Williams order of stability constants of transition metal ions for a given ligand, and with the tendency of first row transition metal ions to quench fluorescence from a bound fluorophore.

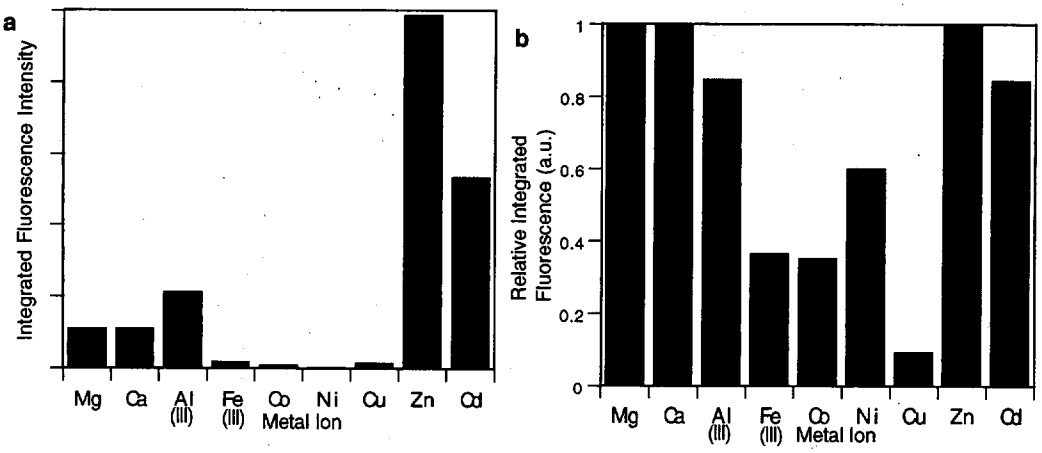


Figure S4. Fluorescence from ligand **9** (1 μM, in 50 mM HEPES, pH 7.0, 150 mM NaCl) (a) on addition of a variety of metal ions (10 μM). (b) on addition of 10 μM $ZnCl_2$, followed by 10 μM of a competing metal ion.

Preparation and Imaging of Cells Incubated with Oxn Derivatives

Mouse fibroblasts (NIH/3T3 or NR6) were plated onto 4-well chamber slides (LabtechII, Nalge Nunc) and grown to 60 – 80% confluency in media (NIH/3T3: high glucose DeltaMem media containing 10% FBS and penicillin/streptomycin (100 unitseach/ml) and NR6: Alpha Mem media containing 7.7% FBS and Geneticin) at 37 °C under an atmosphere of 5% carbon dioxide.

Toxicity studies were performed with probes (100 μM – 2 nM) in media at 37 °C. After 18 hours cells were unaffected at all but the highest concentrations (at or above 25 μM). It is likely that the organic solvent from the stock probe solution (at or above 1.25% in the incubation media) becomes influential at these concentrations.

Fluorescence loading studies were performed at 21 °C or 37 °C, using metal free, phenol red free, Hank's balanced salt solution, containing 1 g/ L glucose (GIBCOBRL). For imaging purposes, cells were left in Hank's buffer containing **9** or the loading solution was replaced by fresh Hank's solution. (In the second case, some leaching of probe back into the loading buffer was observed during subsequent microscopy.) After incubation with **9** (1 – 10 μM), cells were washed with fresh buffer solution before further incubation with competing ligands such as TPEN or EDTA (20 μM). Loss of punctate staining patterns was observed in cells treated with TPEN, but not with cells treated with EDTA, Figure S5. Fainter, non-localized fluorescence staining may be due to tight, nonspecific interactions of the probe with enzyme-bound zinc, or with weak association with Ca(II) or Mg(II), Figure S4.

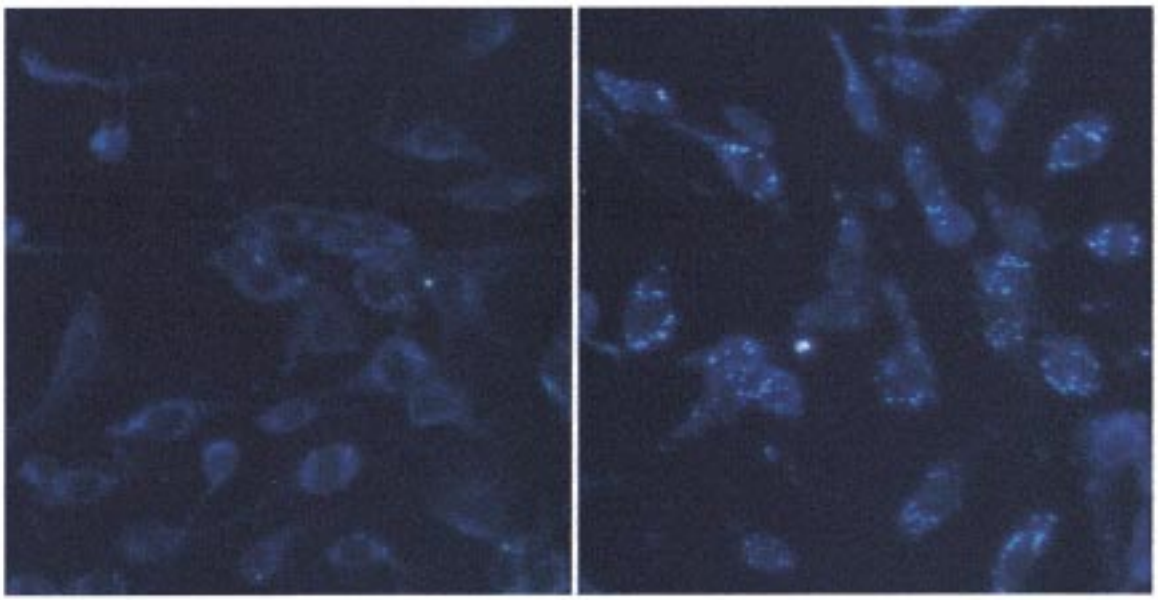


Figure S5. NIH/3T3 mouse fibroblasts incubated with **9** (5 μM, 30 min, 21 °C) followed by subsequent incubation with TPEN (image a, 20 μM, 30 min, 21 °C) or EDTA (image b, 20 μM, 30 min, 21 °C). Residual fluorescence may reflect the hydrophobic probe associating tightly but non-specifically with zinc in a variety of sites such as enzymes, or may be due to some degree of 'cross talk' with intracellular Ca^{2+} and Mg^{2+}.

Incubation of fibroblasts with **7** resulted in faint fluorescence staining, while incubation with **8** resulted in no observable fluorescence labeling. These observations are

consistent with the relative hydrophobicities of **7-9**: **9** > **7** > **8**, and thus may reflect the efficiency with which each compound passes through the cell membrane.

Experiments were monitored using an Olympus IX-50 microscope, fitted with a standard filter set wide band UV-excitation (BP = 330-385nm) filter set. A 12% transmittance filter was used to attenuate the incident light, provided by a mercury lamp. Images were obtained using a DVC-1300 RGB colour digital camera and XCAP-Lite software. Images were trimmed to size for publication using Adobe Photoshop3.0. No attempt was made to match the color of the labeled cells in the printed images to the observed color (which appeared blue-green to the eye).

Supporting References:

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